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Administration of low dose methamphetamine 12 h after a severe traumatic brain injury prevents neurological dysfunction and cognitive impairment in rats

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ABSTRACT

We recently published data that showed low dose of methamphetamine is neuroprotective when delivered 3 h after a severe traumatic brain injury (TBI). In the current study, we further characterized the neuroprotective potential of methamphetamine by determining the lowest effective dose, maximum therapeutic window, pharmacokinetic profile and gene expression changes associated with treatment. Graded doses of methamphetamine were administered to rats beginning 8 h after severe TBI. We assessed neuroprotection based on neurological severity scores, foot fault assessments, cognitive performance in the Morris water maze, and histopathology. We defined 0.250 mg/kg/h as the lowest effective dose and treatment at 12 h as the therapeutic window following severe TBI. We examined gene expression changes following TBI and methamphetamine treatment to further define the potential molecular mechanisms of neuroprotection and determined that methamphetamine significantly reduced the expression of key pro-inflammatory signals. Pharmacokinetic analysis revealed that a 24-hour intravenous infusion of methamphetamine at a dose of 0.500 mg/kg/h produced a plasma C_{max} value of 25.9 ng/ml and a total exposure of 544 ng/ml over a 32 hour time frame. This represents almost half the 24-hour total exposure predicted for a daily oral dose of 25 mg in a 70 kg adult human. Thus, we have demonstrated that methamphetamine is neuroprotective when delivered up to 12 h after injury at doses that are compatible with current FDA approved levels.

Introduction

Currently there are no approved therapeutic interventions available to prevent cognitive and behavioral deficits following traumatic brain injury (TBI) (Beauchamp et al., 2008). The development of viable treatments has been hindered by the fact that TBI represents a heterogeneous injury that activates multiple neuropathological pathways (Beauchamp et al., 2008; Dolan et al., 2012; Elder et al., 2010; Martin et al., 2008; Okie, 2005). During the primary injury phase, there is a rapid, uncontrolled release of glutamate that leads to calcium dysregulation, the production of reactive oxygen species (ROS), reactive nitrogen species (RNS), lipid peroxidation products, the release of prostaglandins, and the generation of nitric oxide (NO) (Pitkanen et al., 2009; Schober et al., 2012; Shultz et al., 2012; Stoica and Faden, 2010; Zhang et al., 2005). Consequently, these molecules induce microglial activation (Blaylock and Maroon, 2011; Block et al., 2007; Brown and Neher, 2010). Microglia are key mediators of both inflammatory responses and glutamate release (Brown and Neher, 2010). When activated in the presence of excessive glutamate, microglia exhibit a neuro-destructive phenotype and secrete pro-inflammatory cytokines such as interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-alpha), and interleukin-1 (IL-1) (Block et al., 2007; Brown and Neher, 2010; Stoica and Faden, 2010). The release of these cytokines induces a cascade of neuro-inflammation that perpetuates glutamate excitotoxicity and microglial activation eventually leading to neuronal loss and permanent neurological dysfunction (Blaylock and Maroon, 2011; Stoica and Faden, 2010).

Thus, survivors of severe TBI are likely to experience a degree of lasting neurological impairment, a permanent reduction in cognitive abilities, and psychological disturbances (Daneshvar et al., 2011; Dempsey et al., 2009). In the United States, 1.7 million individuals suffer from a TBI every year (Prevention C.f.D.C.a, 2006; Reeves and Panguluri, 2011; Saulle and Greenwald, 2012). Clearly, there is a crucial unmet need to develop novel, effective therapies that can be administered

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within a clinically relevant therapeutic window following TBI. We have recently published data demonstrating that administration of low dose methamphetamine following severe TBI resulted in improved functional and cognitive outcomes (Rau et al., 2012). In this previous study, we administered methamphetamine beginning 3 h after a severe TBI. While promising, a 3-hour delay to treatment does not represent a clinically relevant time frame. Therefore, in the present study we examined the therapeutic potential of methamphetamine at later time points. In addition, we more thoroughly characterized the pharmacology of methamphetamine by performing a dose response study and pharmacokinetic analysis. Finally, we examined critical gene expression changes in an effort to understand the potential molecular mechanisms of methamphetamine-mediated neuroprotection.

Methods

The Institutional Animal Care and Use Committee at The University of Montana approved all procedures in these studies. Adult, male, Wistar rats (350–500 g) were obtained from Charles River Laboratories (Wilmington, MA) and housed in filtered isolator boxes with a 12-hour light/dark schedule and ad libitum access to food and water. The lateral fluid percussion injury procedure was performed as we have previously published (Rau et al., 2012). Briefly, animals were deeply anesthetized using 2–4% isoflourane. A 5 mm trephination was made over the right hemisphere equidistant from the lambda and the bregma as we previously described (Rau et al., 2012). Animals were given a 20 ms fluid pulse to the dura at 1.9–2.3 ATM of pressure. Approximately 25% mortality was observed with these pressures. All animals became apneic after injury and were manually ventilated with supplemented O_2 until normal breathing resumed. At 7.5 or 11.5 h post-injury, an Alzet osmotic pump (Alzet corp; 2001D; 8.4 μL per hour) containing a methamphetamine solution designed to deliver 0.500, 0.250, or 0.250 mg/kg/h for 24 h was surgically implanted into the inguinal crease and connected to the femoral vein through a catheter as we previously described (Rau et al., 2012). The catheter (Scientific Commodities; Lake Havasu, AZ) from the pump was pre-loaded with a solution of 50% dextrose/50% heparin to prevent clotting. The length of the catheter and inner diameter were calculated to ensure methamphetamine delivery to the end of the catheter 30 min after insertion into the animal. At 8 or 12 h post injury, the animals were lightly anesthetized and a bolus dose of saline or methamphetamine was injected into the tail vein to coincide with the beginning of the pump delivery into the femoral vein. Bolus dosing for the 0.500 mg/kg/h dose was 0.425 mg/kg, 0.212 mg/kg for the 0.250 mg/kg/h dose, and 0.106 mg/kg for the 0.125 mg/kg/h dose. Saline and sham treated animals underwent the same procedure receiving pre-warmed saline. 72 h after injury the animals were re-anesthetized and the Alzet pumps were removed (Rau et al., 2012).

Neurological severity scoring

Neurological severity scoring (NSS) was performed as previously described (Rau et al., 2011, 2012). NSS and foot fault assessments were conducted on days 1, 7, 14, 21, 30, and day 40. Animals were scored from 0–16 with 16 indicating maximal impairment. Scoring criteria for a severe TBI was 16-10, Animals scoring 9 or less on day 1 were excluded as a moderate/mild injury.

Foot faults assessments

Foot fault assessments were conducted as previously described (Rau et al., 2012). Briefly, rats were set on an elevated grid. With each weight-bearing step, the paw may fall or slip off the wire grid. Each time the left forelimb (affected by damage to the right hemisphere) missed a placement on the wire rack it was recorded as a foot fault. The total number of steps (weight bearing movement of the right forelimb) that the rat used to cross the grid was counted, and the total number of foot faults for each forelimb was recorded.

Assessment of cognitive function

The Morris water maze (MWM) was used to assess the impact of methamphetamine on cognitive function (learning and memory) following TBI. The assessment procedure was performed as previously published (Rau et al., 2012). Pre-acclimation began on day 39 post-injury. The training phase began on day 40 post-injury, and the probe trial was conducted on day 45 post-injury. The water temperature was maintained at a constant 19 °Celsius with the clear plexiglas platform 2 cm below the water level. All data was recorded and analyzed using Anymaze software connected to a Logitech camera. All data sets were analyzed by a blinded researcher. There were no significant differences in swim speeds between any of the animals.

Immunohistochemistry

Forty-six days after TBI, rats were deeply anesthetized and perfused with 4% paraformaldehyde (PFA) fixative. Brains were post-fixed for 24 h at 4 °C in 4% PFA and divided into 2 mm coronal sections using a rat brain matrix. Slices were processed, paraffin embedded and sectioned at 7 μm. A 1 in 10 series from each rat was surveyed to identify location relative to Bregma. Sections were mounted at Bregma −3.3, deparaffinized and rehydrated. Antigen retrieval by treatment in 0.1 M citric acid pH 6 was performed, and sections were stained for a pan-axonal neurofilament marker (NF312 Covance, Princeton NJ) at a dilution of 1:1500 overnight at 4 °C in a humid chamber. Slides were washed with PBS 3 × 5 min and incubated with AlexaFluor 488 Goat anti Mouse IgG (Invitrogen, Life Technologies, Grand Island, NY) at a dilution of 1:300 for 1 h at room temperature in the dark. After rinsing in PBS 3 × 5 min nuclei were counterstained with DAPI (Invitrogen) diluted to 1 μg/ml in PBS for 5 min. Sections were rinsed in PBS, rinsed briefly in ddH_2O and coverslipped using FluorSave mounting media (Calbiochem, Darmstadt, Germany). Adjacent sections at Bregma −3.3 were deparaffinized, rehydrated, and permeabilized in 0.1% TritonX100 in PBS for 20 min. Following a PBS rinse slides were stained for 488 NeuroTrace (Molecular Probes, Life Technologies, Grand Island, NY) at a dilution of 1:400 overnight at 4 °C to label neurons. After NeuroTrace labeling, sections were rinsed in PBS 3 × 5 min and counterstained with DAPI as above. All sections were imaged on an Olympus Fluoview FV1000 confocal microscope (Olympus, USA), capturing the single brightest plane of fluorescence. For NF312 stained slides, images were captured at 40× (1.5 zoom) for the CA3 region of the hippocampus. Integrated Optical Density (IOD, a measure of area multiplied by the average density of staining) and axon length (a measure of total length of all one pixel thick open branches) of NF312 staining was measured on the injured hemisphere of the brain sections using ImagePro 6.2 software (ImagePro, Bethesda MD). The sum of the IOD or sum axon length from five saline, six 8 h and four 12 hour methamphetamine treated rats were measured. Treatment group averages of sum IOD or axon length and standard errors of the mean were calculated. Positive staining levels were identified by setting threshold levels from control slides reacted with secondary but no primary antibody. For the NeuroTrace stained slides images were captured at 20× (1.5zoom) in the CA1 region of hippocampus. Two sections per rat were imaged and dead cells were counted manually using ImagePro software. Live cells were round and contained NeuroTrace labeled (green) cytoplasm and blue (DAPI labeled) nuclei. Dead cells were very bright cells with condensed cytoplasm and nuclei and altered morphology. Average dead cells per rat and an average number of dead cells per treatment group were calculated. Four saline and four 8-hour methamphetamine and five 12 hour methamphetamine rats were counted and group averages and standard errors of the mean calculated.

RNA isolation/gene array protocol

Three biological replicates were used for each experimental group and each replicate was run in triplicate. Total RNA was isolated from the rat ipsilateral cortex utilizing Trizol LS (Invitrogen) according to the manufacturer's protocol. To remove any contaminating chemicals

that could affect downstream analysis, RNA was further purified using the Qiagen RNeasy MinElute Cleanup Kit according to the manufacturer's protocol. RNA was quantified using a Nano Drop spectrophotometer. RNA was considered acceptable if the 260/280 and 260/230 ratios were 2.0 or greater. A 1 μg aliquot of total RNA was reverse transcribed to cDNA using Qiagen's RT2 First Strand Kit. One half of the cDNA reaction was mixed with 675 μl of Qiagen's 2X RT SYBR Green Mastermix and 624 μl H2O. This was then added to a custom array panel (SA Biosciences) and cycled according to the manufacturer's suggested qPCR protocol on a Bio-Rad iQ5 thermocycler. Values were normalized for GAPDH. For data analysis, the ΔΔCt method was used with the aid of the manufacturer's online software suite RT2 Profiler PCR Array Data Analysis version 3.5. Fold-changes were calculated and results compared to normal controls. Please note: (2^(- Delta Delta Ct)) is the normalized gene expression (2^(- Delta Ct)) in the test sample divided the normalized gene expression (2^(- Delta Ct)) in the Control Sample. Fold-regulation represents fold-change results in a biologically meaningful way. Fold-change values greater than one indicate a positive- or an up-regulation, and the fold-regulation is equal to the fold-change. Controls for this experiment underwent the trephination surgery but did not receive a TBI.

Measurement of methamphetamine from plasma

Jugular catheterized adult male Wistar rats were purchased from Charles River Laboratories. Rats were implanted with an Alzet pump (Alzet Corp., 2001D, 8.4 μl per hour) and a femoral vein catheter as previously described (Rau et al., 2012). Pump delivery was calculated based on pump rate and volume contained within the catheter. Based on the catheter inner diameter (ID) of 0.635 and a pump rate of 8.4 μl per hour +/−0.2 μl a catheter length of 2.25 cm delivered methamphetamine to the femoral vein at 1 h post-implantation. A total of six blood draws (500 μl each) were performed on each animal. Animals were anesthetized using 1–3% isoflurane and maintained at a plane of light sedation throughout the blood draw. Collected blood tubes were placed on ice and centrifuged immediately. Samples were spun at 1300 ×*g* for 10 min. Supernatants were immediately pulled off and placed in Eppendorf tubes. Samples were immediately frozen at −80 °C until processing and testing. Plasma samples were processed using the State of Montana crime lab extraction protocol and analyzed using LC-MS at the Missoula, Montana laboratory (Cravey, 1982; S. B., 1996).

Statistical analysis

All data was analyzed using Prizm software. To determine Gaussian (normal) distribution a Kolmogorof–Smirnov test was performed on all data sets. Appropriate parametric analysis was performed on data sets containing two groups using an unpaired, one-tailed *T*-test (CI = 95%). Analysis on data sets with more than 2 groups was done using one-way ANOVA with Tukey's post-hoc to determine statistical significance between groups. A $p < 0.05$ was considered significant.

Results

Methamphetamine improves behavioral outcomes when administration is delayed until 8 h after injury

We previously demonstrated that methamphetamine provides robust improvement in both behavioral and cognitive function when administration occurs within 3 h after severe TBI. However, treatment within 3 h is not clinically achievable in many cases. Therefore, we further characterized the neuroprotective potential and pharmacological profile of methamphetamine by conducting a dose response study with administration being delayed until 8 h after injury. Rats received a severe TBI and, 8 h later, were given an i.v. bolus injection of saline or methamphetamine (0.42, 0.21, or 0.10 mg/kg), followed by i.v. infusion for 24 h with saline or methamphetamine (0.50, 0.25, or 0.12 mg/kg/h), respectively. NSS and foot fault assessments were conducted on days 1, 7, 14, 21, 30 and 40 post-injury (Rau et al., 2012). Administration of methamphetamine at the 0.500 mg/kg/h or 0.250 mg/kg/h dose produced significant improvements in NSS on days 14, 21, 30 and 40 post injury (Fig. 1A). However, a significant improvement in neurological scoring for animals treated with 0.500 mg/kg/h dose was observed as early as 7 days post-injury, suggesting that the higher dose may have a slightly greater effect on early recovery from the injury. Animals receiving the 0.125 mg/kg/h dose had a significant improvement on day 14 post-injury however this effect did not persist. On days 1, 7, 21, 30 and 40, post-injury NSS for the 0.125 mg/kg/h treatment group did not differ from saline treated animals.

Dexterity, fine motor control and forepaw sensation was assessed using the foot fault test (Rau et al., 2012). Animals were examined on days 1, 7, 14, 21, 30, and 40 post-injury. Unlike the NSS assessment, all doses of methamphetamine improved performance in the foot fault assessment by day 14 post-injury (Fig. 1B). However, as with the NSS scoring, the 0.500 mg/kg/h dose showed improvement earlier (at 7 days post-injury) than the other doses did.

Methamphetamine improves cognitive outcomes when administration is delayed until 8 h after injury

The dose response effect of methamphetamine on cognitive performance following severe TBI was assessed using the Morris water maze (MWM). Dosing rats with 0.500 mg/kg/h or 0.250 mg/kg/h beginning 8 h after injury significantly reduced the time required to locate the

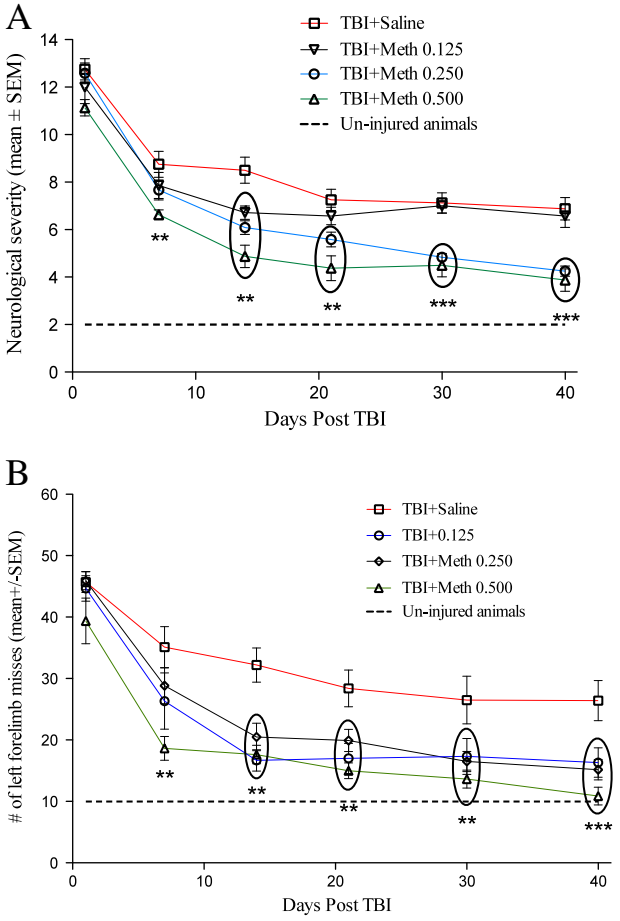

Fig. 1. Behavioral assessments following 8 hour delay of methamphetamine administration. Time course values are shown for (A) Neurological Severity Scores (NSS) and (B) foot fault assessments over 40 days for rats treated with saline (red line), 0.125 mg/kg/h (black line), 0.25 mg/kg/h (blue line) or 0.500 mg/kg/h (green line). The dashed line indicates typical NSS and foot fault scores for uninjured rats. n = a minimum of 8 animals per group. ** = $p < 0.01$, *** = $p < 0.001$ relative to saline treated controls.

escape platform (Fig. 2A). A significant improvement in learning was observed on training days 3, 4, and 5 for rats treated with the 0.500 mg/kg/h dose. Rats treated with this dose behaved similar to uninjured rats by the third training day. However, rats treated with 0.250 mg/kg/h did not show significant improvement over saline treated controls until the fifth training day. In contrast, the 0.125 mg/kg/h treated rats showed no significant improvement over saline controls on any of the days tested indicating this dose failed to improve learning following severe TBI.

After completion of the five-day training period, we used a probe trial to assess memory function. Using this assessment of spatial memory, we found that injured rats treated with either 0.500 mg/kg/h or 0.250 mg/kg/h methamphetamine spent significantly greater time in the target quadrant searching for the escape platform (Fig. 2B) and significantly less time (~25%) in the thigmotaxia zone (Fig. 2C) compared to saline treated, injured animals. The thigmotaxia zone is the circular area around the outer rim of the tank and was not included in the four search quadrants. Animals that spend time in this zone are not actively searching or using visual cues and thus swim time spent in the thigmotaxia zone represents a random search pattern that does not involve active spatial memory recall. Animals treated with 0.125 mg/kg/h methamphetamine did not differ from saline treated, injured animals in the amount of time they spent in the target quadrant or the thigmotaxia zone, indicating that the 0.125 mg/kg/h dose of methamphetamine had no effect on memory improvement after TBI.

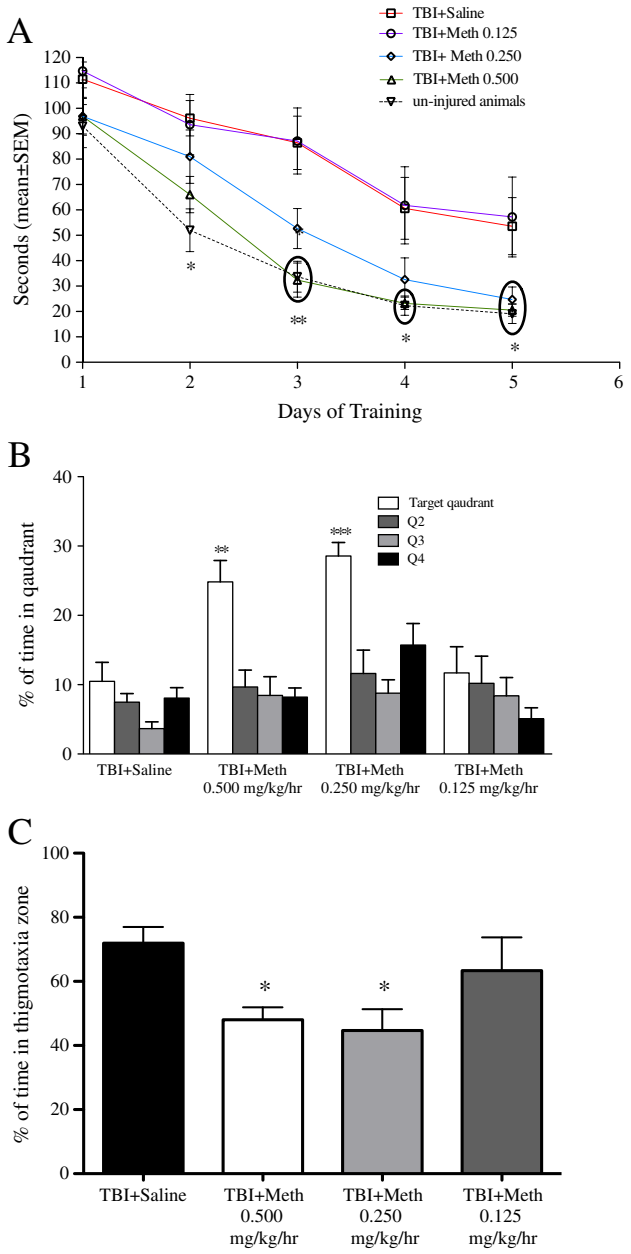

Fig. 2. Treatment with methamphetamine improves learning and memory after severe TBI. (A) Shows latency times over 5 training days for rats treated with saline (red line), 0.125 mg/kg/h (black line), 0.250 mg/kg/h (blue line) or 0.500 mg/kg/h (green line) beginning 8 h after severe TBI. The dotted black line indicates the latency times for uninjured control rats. (B) Shows the percent time rats spent in each quadrant of the maze searching for the escape platform. The target quadrant (white bars) is where the escape platform had previously been located. Non-target quadrants were Q2-4. (C) Shows the percent of time that rats from each treatment group spent within the thigomataxia zone. n = 8 animals per group. * = $p < 0.05$, ** = $p < 0.01$ relative to saline treated controls.

Methamphetamine is neuroprotective when administration is delayed up to 12 h post TBI

To further characterize the pharmacology of methamphetamine as a potential neuroprotective agent and define possible parameters of clinical application, we examined the therapeutic window of treatment. To accomplish this, we delayed delivery of methamphetamine until 12 h after severe TBI. We again used NSS and foot fault tests to assess

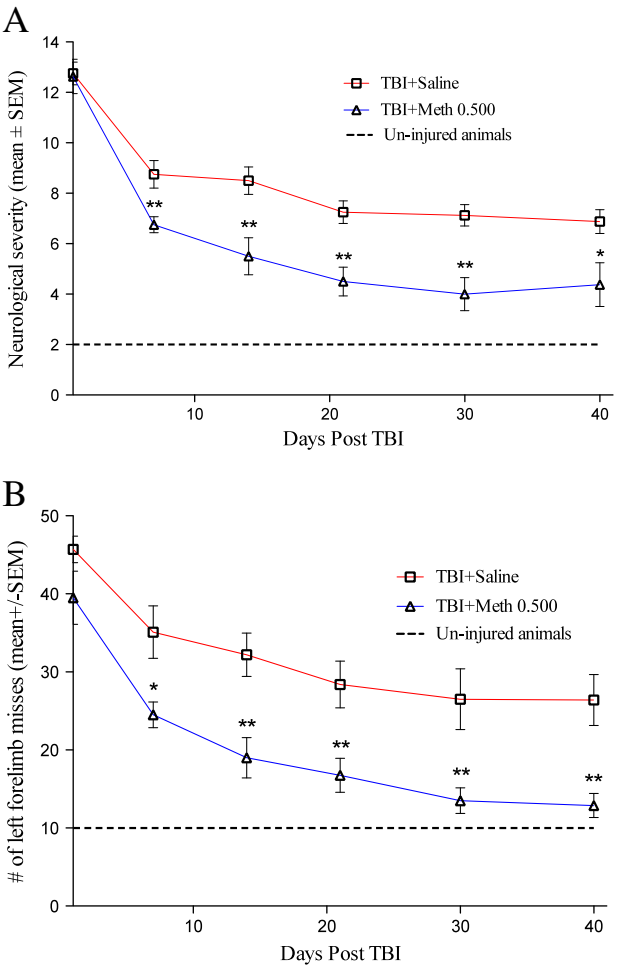

Fig. 3. Methamphetamine administered 12 h after TBI reduced neurological severity scores. Time course values are shown for (A) Neurological Severity Scores and (B) foot fault assessments over 40 days for rats treated with saline (red line), or 0.500 mg/kg/h (blue line). The dashed line indicates typical NSS and foot fault scores for uninjured rats. n = a minimum of 8 animals per group. * = $p < 0.05$, ** = $p < 0.01$, relative to saline treated controls.

functional behavior. We observed a significant improvement in both NSS and foot fault measurements within 7 days following delayed administration (Fig. 3).

Spatial learning and reference memory were again assessed in the MWM (Rau et al., 2012). As with the NSS and foot fault testing, delaying administration of methamphetamine until 12 h after severe TBI resulted in significantly improved performance in the MWM. Similar to the 8-hour delivery study, we observed a robust improvement in memory and a significant reduction in the time spent in the thigmotaxia zone (Fig. 4). However, rats treated with methamphetamine beginning at 12 h after injury did not show a significant improvement in latency times until the fifth day of training. This represents a substantial shift in the learning curve relative to the 8-hour study and indicates that delaying methamphetamine treatment to 12 h post-TBI reduces improvement in this outcome.

Methamphetamine treatment reduces neuronal loss and preserves axon density after severe TBI

Immunofluorescent analysis was conducted on paraffin embedded brain sections from the injury area. Sections were prepared at 46 days after injury. As previously described by Ooigawa et al. (2006) we utilized a fluorescent Nissil stain (NeuroTRACE), to label both live and dead neurons in injured animals treated with saline or methamphetamine (Ooigawa et al., 2006). We found that treating rats with 0.500 mg/kg/h methamphetamine beginning at 8 or 12 h after severe TBI significantly reduced neuronal loss in the CA1 region of the hippocampus compared to injured, saline treated control animals (Fig. 5). Diffuse axonal injury is often observed following TBI. Therefore, we assessed the potential of methamphetamine to preserve axonal density. Using a pan-antibody for neurofilament, we found that delivering methamphetamine at the 0.500 mg/kg/h dose, beginning 8 h after injury, significantly preserved neurofilament staining within the CA3 region of the hippocampus when compared to saline treated control animals (Fig. 6). However, there was no significant difference in neurofilament staining in the hippocampus when treatment was delayed until 12 h post-injury.

Pharmacokinetics at a therapeutic dose of methamphetamine

To more fully characterize the pharmacology of methamphetamine we examined the pharmacokinetic profile of the highest therapeutically effective dose tested. A 24-hour infusion of methamphetamine at 0.500 mg/kg/h for 24 h generated a C_{max} of 25.9 ng/ml and an exposure of 17 ng/ml/h over a 32-hour period. This produced a total drug exposure of 544 ng/ml. The FDA recommended oral dose for treating ADHD is up to 25 mg daily (Administration F.a.D, 2007). Based on this dosing regimen, we calculated a 24-hour total drug exposure of 998 ng/ml, for a 70 kg human receiving a daily oral dose of 25 mg. We also analyzed the bioactive metabolite D-amphetamine, and found that exposure to 0.500 mg/kg/h methamphetamine generated a C_{max} of 13.3 ng/ml and a total drug exposure over 32 h of 252 ng/ml (Fig. 7).

Methamphetamine significantly alters the expression of genes involved in multiple pathophysiological pathways

We previously reported that methamphetamine mediates neuroprotection through dopamine-dependent activation of a PI3K-AKT signaling pathway (Rau et al., 2011). We have further demonstrated that methamphetamine significantly reduced apoptosis after severe stroke and TBI (Rau et al., 2011). In addition to the histological evidence of neuroprotection presented in this study, we wanted to further define the potential mechanisms of methamphetamine-mediated neuroprotection. Therefore, we conducted a gene array analysis to screen for candidate genes involved in the pathological processes induced by severe TBI. As Table 1 indicates, methamphetamine treatment prevented the increased expression of several key genes involved in inflammatory signaling. Many of the largest increases (when compared to sham, un-injured animals) we observed in gene expression involved pro-inflammatory molecules. For example, the chemokine CCL2 (11 fold increase, $p = 0.0004$), the Toll-like receptor signaling protein Myd88 (3 fold increase, $p < 0.0001$) and the pro-inflammatory cytokine IL1β (4.6 fold increase, $p = 0.005$) were all significantly elevated in saline treated TBI injured

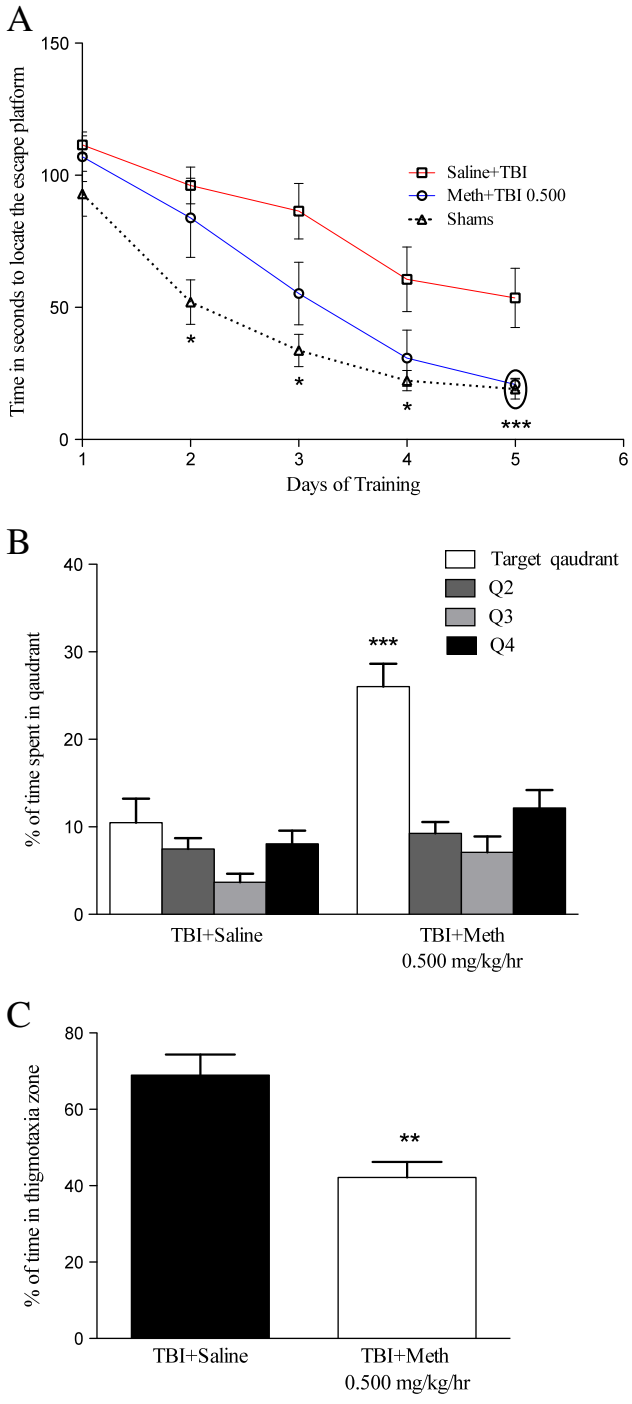

Fig. 4. Treatment with low dose methamphetamine improved learning and memory when delivered 12 h after a severe TBI. (A) Shows latency times over 5 training days for rats treated with saline (red line), or 0.500 mg/kg/h (blue line) beginning 12 h after severe TBI. The dotted black line indicates the latency times for uninjured control rats. (B) Shows the percent time rats spent in each quadrant of the maze searching for the escape platform. The target quadrant (white bars) is where the escape platform had previously been located. Non-target quadrants were Q2-4. (C) Shows the percent of time rats from each treatment group spent within the thigomataxia zone. n = 8 animals per group. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ relative to saline treated controls.

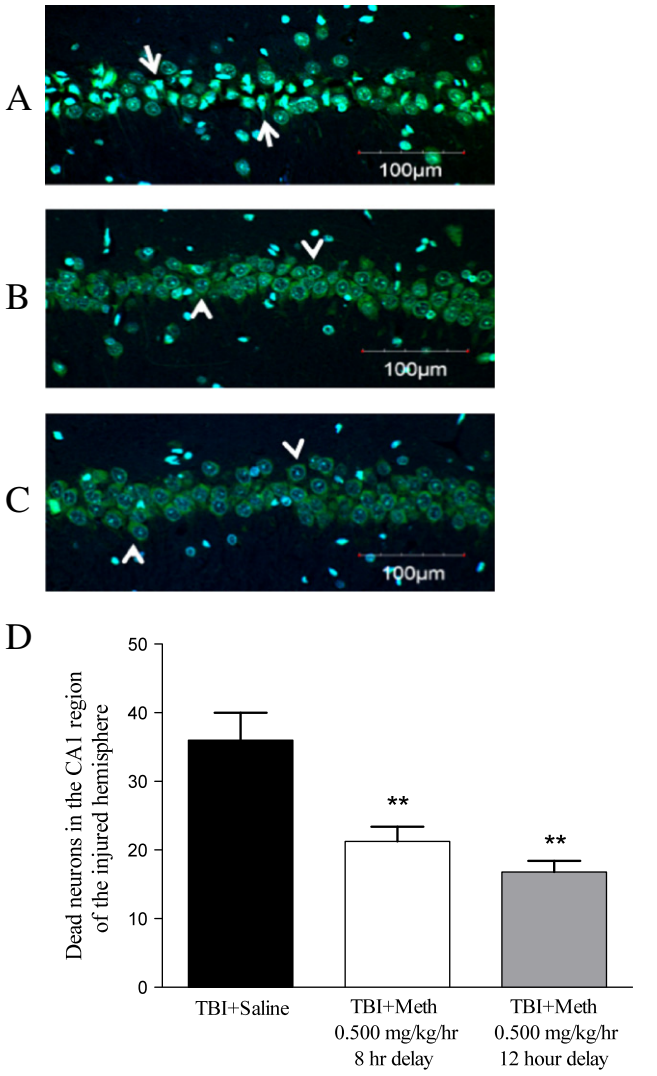

Fig. 5. Methamphetamine is neuroprotective in the CA1 region of the hippocampus at 46 days after severe TBI. Representative micrographs of the CA1 region at Bregma −3.3 show increased numbers of dead cells in saline treated TBI animals (A) compared with rats treated with 0.500 mg/kg/h methamphetamine at 8 h post TBI (B) or at 12 h post-TBI (C). Nuclei are stained blue with DAPI. Dead and dying neurons stain brightly green with NeuroTrace with little or no nuclei (arrows in a). Normal neurons have blue nuclei and rounded cell morphology with a large cytoplasm that stains less intensely green (arrowheads in b and c). (D) Shows graphically the average values for each treatment group. n = 4 animals per group. ** = $p < 0.01$.

animals. Conversely, we did not observe significant changes in the expression of CCL2, Myd88 or IL1β in the methamphetamine treated rats. However, methamphetamine treatment did induce a significant increase in the anti-inflammatory chemokine CXCL12 (2.2 fold, $p = 0.0001$) while saline controls showed no significant change. Finally, we observed at least a two-fold increase in the expression of corticotropin releasing hormone ($p = 0.0006$), and neurotensin ($p = 0.008$) in methamphetamine treated rats compared to a 1.7 fold decrease ($p = 0.002$) in corticotropin releasing hormone and no significant change in neurotensin in saline treated controls.

Discussion

In this study we have established 0.250 mg/kg/h as the lowest effective dose of methamphetamine that significantly reduces neurological dysfunction and cognitive impairment when delivered 8 h after a severe

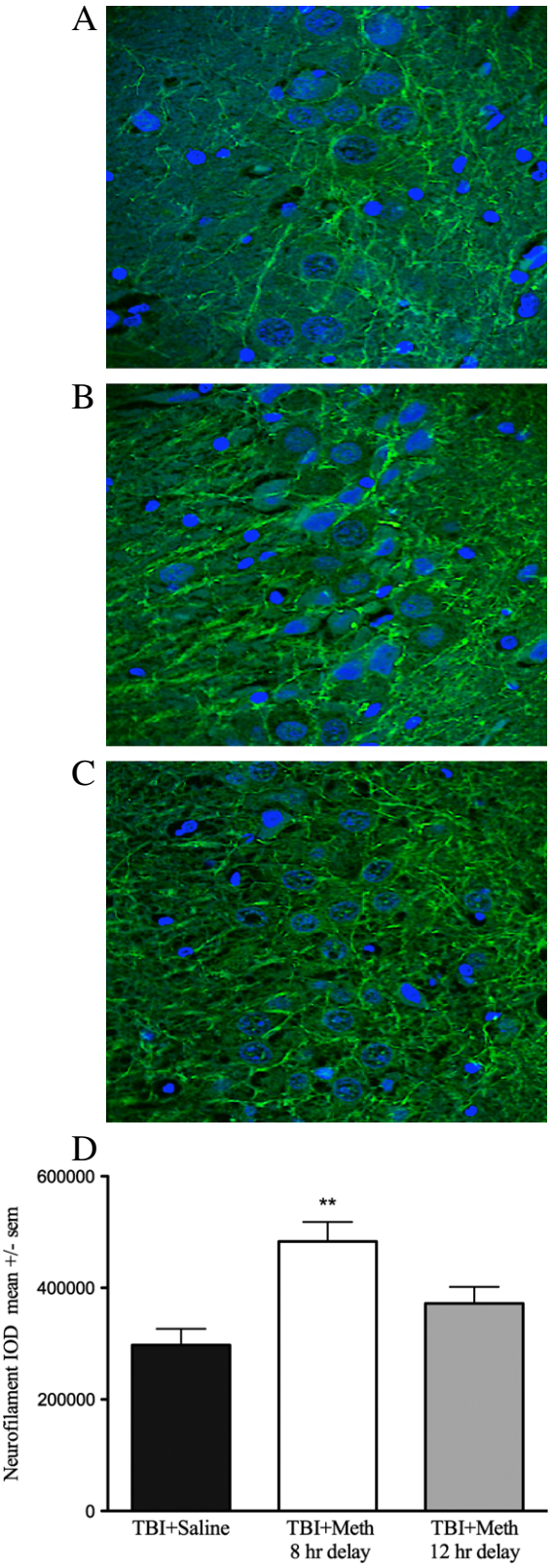

Fig. 6. Methamphetamine preserves axonal structure. Representative images of the CA3 region show decreased neurofilament staining in saline treated TBI injured animals (A) compared to TBI rats treated with methamphetamine at 8 h after injury (B). Animals treated with methamphetamine beginning 12 h post TBI did not show a significant difference from saline treated TBI animals (C). Quantification of neurofilament staining showed significantly greater axonal staining (d) in rats treated with methamphetamine beginning 8 h after injury, but not at 12 h, (D). n = minimum of 4 rats per group. * = $p < 0.05$, ** = $p < 0.01$.

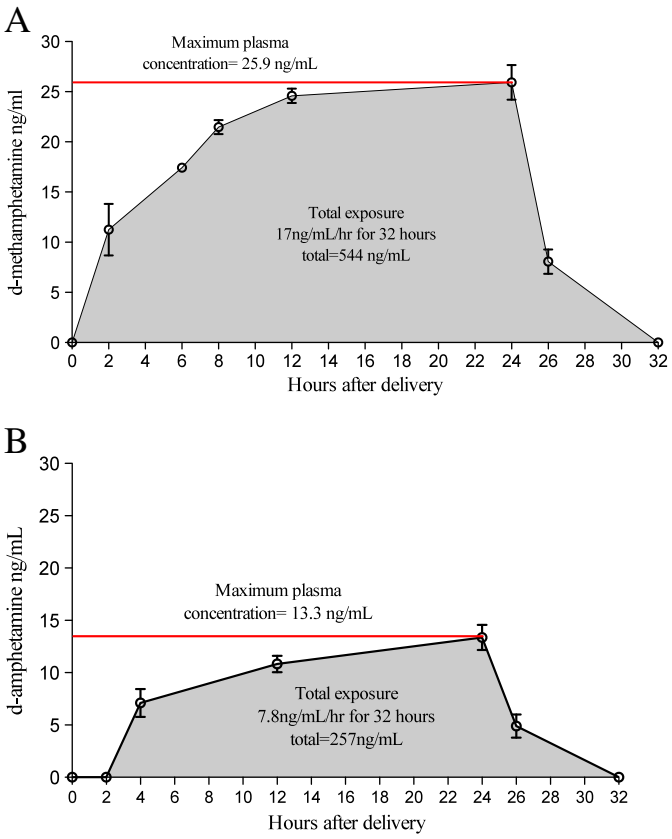

Fig. 7. Pharmacokinetics of D-methamphetamine and its metabolite, D-amphetamine. The concentrations of methamphetamine (A) and amphetamine (B) detected in the plasma of rats treated with methamphetamine at a dose of 0.500 mg/kg/h for 24 h are shown.

TBI. We further established that methamphetamine produced significant behavioral and cognitive improvements when a dose of 0.500 mg/kg/h is delivered up to 12 h after a severe TBI. Immediately after TBI, transient mitochondrial swelling occurs, which after 12 h, leads to irreversible diffuse axonal injury (Blaylock and Maroon, 2011; Saulle and Greenwald, 2012; Stoica and Faden, 2010; Thompson et al., 2005). Thus, the inability of methamphetamine to preserve axons after a 12-hour delay may directly correlate with these axonal studies. It may also explain why delaying methamphetamine treatment until 12 h after injury had less of an impact on the learning assessments. Despite the loss of neurofilament staining in the CA3 region, animals in the 12-hour delay group still demonstrated a significant improvement in memory. The disparity between the learning and memory outcomes may be explained by our observation that the 12-hour delay group still had a significant preservation of CA1 pyramidal neurons, which are critical in memory consolidation (Wittenberg et al., 2002). This suggests that low dose methamphetamine can be delivered up to 12 h after injury and still protect highly vulnerable neurons within the hippocampus.

Table 1
Gene expression analysis in TBI rats.

	Fold change	
Gene	Saline/normal	Methamphetamine/normal
CCL2	11 (0.0004)	NSC
MyD88	3 (0.0001)	NSC
IL1b	4.6 (0.005)	NSC
CXCL12	NSC	2.2 (0.0001)
CRH	−1.7 (0.002)	2 (0.0006)
neurotensin	NSC	2 (0.008)

NSC = no significant change. P values relative to normal (uninjured) rats are shown in parenthesis.

Of particular interest in this study was our finding that methamphetamine improved NSS scoring and foot faults but reduced cell death and axonal loss only in the hippocampus. A comprehensive literature search provides a spectrum of contradicting outcomes in the context of motor function relative to cortical injury in the rat LFP model. Selected LFP studies have shown a correlation between a reduction in cortical lesion volume and motor function improvement (Luo et al., 2013; Otani et al., 2007). In contrast, other studies have shown improved motor behavior with no decrease in cortical lesion volume (Lippert-Gruener et al., 2007). Further studies have reported neuroprotective treatments that improved lesion volume but did not improve motor function (Marklund et al., 2001).

Others have shown a correlation between reduced hippocampal damage following LFP-induced TBI and improved motor function (Lee et al., 2004). Similar to our studies, Lee et al. (2004) only reported pathological alterations in the hippocampus. Supporting this observation, Gheysen et al. (2010) presented evidence that the hippocampus contributes to early and late stage motor sequence learning that is critical to performance of motor function tasks (Gheysen et al., 2010). Further supporting the role of the hippocampus in motor tasks Grossberg and Merrill (1996), found that the hippocampus plays a key role in controlling adaptive timing and spatial orientation which are two key elements of balance and coordination, both of which are tested in the NSS scoring and foot fault analysis (Grossberg and Merrill, 1996; Gorchetchnikov and Grossberg, 2007). Specifically, Grossberg found clear evidence that the hippocampal circuits play a key role in cerebellar timing control, spatial orientation, and motor outputs (Grossberg and Merrill, 1996; Gorchetchnikov and Grossberg, 2007; Grossberg and Seidman, 2006).

The LFP model creates a global stress on the brain and generates the coup-contre coup effect found in closed head injuries (Xiong et al., 2012). Interestingly, closed head injuries can produce motor deficits but fail to generate a cortical lesion, (or measurable damage) within the motor cortex (Heitger et al., 2006). Heitger et al. (2006) also found that despite the lack of measureable tissue abnormalities, a mild closed head TBI could produce motor deficits up to 1 year following the injury (Heitger et al., 2006).

Another potential explanation may be linked to white matter track remodeling that occurs after TBI. We previously reported (Ding et al., 2013) that methamphetamine treatment improved foot faults and NSS scores but did not decrease cortical lesion volumes (Ding et al., 2013). However, we did observe a statistically significant increase in white matter track remodeling within the peri-lesional region of the cortex in methamphetamine treated animals that was not present in saline treated controls. Interestingly, this significant change was not observed until 5 and 6 weeks post injury suggesting methamphetamine treatment may have a long-term effect irrespective of the initial lesion core (Ding et al., 2013).

After administration, methamphetamine rapidly concentrates in the brain at significantly higher levels than in the plasma (up to 13 times greater) and induces the immediate release of dopamine, norepinephrine and serotonin (Administration F.a.D, 2007; Krasnova and Cadet, 2009; S. B., 1996). Methamphetamine also blocks the degradation of these neurotransmitters, resulting in prolonged neuronal signaling (Cadet and Krasnova, 2009; Krasnova and Cadet, 2009). When used at very high doses it produces profound euphoria, increased heart rate, respiration, agitation and dopamine mediated neurotoxicity (Cadet and Krasnova, 2009; Krasnova and Cadet, 2009). However, when administered at low doses, methamphetamine produces a more controlled release of catecholamines, capable of activating multiple neuroprotective pathways in the brain (Feeney and Hovda, 1983; Feeney et al., 1981, 1982; Rau et al., 2011, 2012). Currently, methamphetamine is FDA approved for the treatment of ADHD and obesity as an oral daily dose of up to 25 mg (Administration F.a.D, 2007). Under this dosing regimen, the total 24-hour exposure for a 70 kg adult would be twice what we

observed with the highest therapeutic dose (0.500 mg/kg/h) tested in rats. However, recently published data indicates that a single i.v. injection of 0.5 mg/kg methamphetamine in a healthy human test subjects produced a 24 h total exposure similar to what a daily oral dose of 25 mg would produce (Mendelson et al., 2006). This suggests that the current FDA approved dose may more than sufficient to provide neuroprotective efficacy in humans.

A recent study by Vaarmann et al. (2013) suggested that dopamine prevents delayed calcium deregulation and glutamate excitotoxicity (Vaarmann et al., 2013). This is relevant as TBI produces glutamate-mediated excitotoxicity and calcium dysregulation. This effect was demonstrated to be dependent on the activation of D1 and D2 receptors and was enhanced by the addition of a monoamine oxidase inhibitor. We have previously reported that methamphetamine-mediated neuroprotection in a stroke model was dependent on dopamine activation of D1 and D2 receptors (Administration F.a.D, 2007). As a secondary effect, methamphetamine also inhibits monoamine oxidase activity. Thus, methamphetamine may be exerting a neuroprotective effect by preventing delayed calcium deregulation and excitotoxicity. Furthermore, we have also previously demonstrated that delivering low dose methamphetamine increased protein kinase B (AKT) phosphorylation in a dopamine dependent manner (Rau et al., 2011). This finding is also linked to neuroprotection as AKT is a central kinase that blocks multiple pathways of apoptotic death including BAD, Bim, GSK3β, p53 and Fox01 (Rau et al., 2011; Zhang et al., 2005). Further supporting the potential anti-apoptosis effect of AKT phosphorylation, we found that methamphetamine treatment significantly reduced apoptotic neuronal death in the hippocampus.

In addition to apoptosis, TBI induces a cascade of pathophysiology involving a broad spectrum of gene expression changes. We observed large changes in the expression of key genes that influence cerebral inflammation 32 h after TBI. Specifically, methamphetamine treatment significantly reduced cortical expression of three key mediators of inflammation: CCL2, MyD88 and IL1ß.

The pro-inflammatory chemokine CCL2 (MCP-1) increased 11 fold in injured, saline treated animals, whereas treatment with methamphetamine completely blocked this increase. A member of the monocyte chemotactic protein (MCP) family, CCL2 is expressed by astrocytes, macrophages, and reactive microglia, and is one of the most potent chemo-attractants that recruit monocytes to damaged areas (Sozzani et al., 1994a, 1994b). In addition, there is direct evidence that CCL2 contributes to the pathogenesis and development of brain lesions from ischemia and excitotoxic insults, both of which play a role in the development of pathology following TBI (Semple et al., 2010, 2011). Supporting this finding is the observation that CCL2 knockout mice have significantly lower neurological impairment following TBI relative to wild type mice (Lloyd et al., 2008). A rapid increase in CCL2 is observed within human spinal fluid following severe TBI and CCL2 levels remain significantly elevated over controls for up to 10 days post-injury (Semple et al., 2010). Semple et al. (2010) recently reported that increased levels of CCL2 were detected in the serum of patients who died shortly after TBI (Semple et al., 2010). Thus, a direct correlation exists between increased CCL2 levels, and increased neuro-inflammation and neuropathology following TBI. Our findings suggest that methamphetamine mediates neuroprotection in part, through the reduction of this critical neuro-inflammatory mediator.

The concept that methamphetamine treatment reduces post-traumatic inflammation is further supported by the fact that TBI induced a 3-fold increase in the expression of the myeloid differentiation primary response protein 88 (Myd88) gene, while a significant increase in Myd88 expression was absent in the methamphetamine treated rats. Myd88 is a key adaptor protein involved in Toll-like receptor and pro-inflammatory cytokine signal transduction. Activation of Toll-like receptors results in the recruitment of Myd88 and subsequent activation of the transcription activator, nuclear factor kappaB (NF-κB). NF-κB induces the rapid expression of pro-inflammatory molecules including tumor necrosis factor a (TNF-α), IL-1β, interleukin-6 (IL-6), and intracellular adhesion molecule-1 (ICAM-1) (G.Z. Li et al, 2011.; W. Li et al., 2013; Ling et al., 2013). This may explain the 4.6 fold increase we observed in IL-1β after TBI. Li et al. (2011) demonstrated a significant increase in Myd88 positive cells surrounding the TBI injury zone and a positive correlation between the expression of Myd88, NF-κB, and IL-1β (G.Z. Li et al., 2011; W. Li et al., 2013; Zhang et al., 2012). Further supporting the inflammatory role of Myd88 in TBI, Li et al. (2011) found that Myd88 was significantly elevated 72 h after TBI in humans and was actively expressed in neurons, astrocytes, and microglia (G.Z. Li et al., 2011; W. Li et al., 2013). This correlated with a significant increase in TNF-α and IL-1β (W. Li et al., 2013; G.Z. Li et al., 2011).

In our experiments, we observed Myd88, CCL-2, and IL-1β expression levels in methamphetamine treated TBI animals that were not significantly different from uninjured sham controls 32 h after TBI. The exact mechanism(s) of how methamphetamine produced the gene changes we observed are not known, and there are no current studies that link methamphetamine to decreased neuroinflammation. There are studies, however, that suggest a link between dopamine signaling and decreased inflammation. Shao et al. (2012) demonstrated that the astrocytic D2 dopamine receptor (D2R) potently suppressed neuroinflammation in the rodent brain by influencing expression of αB-crystallin (CRYAB) (Shao et al., 2012). Coffeen et al. (2010) demonstrated that brain inflammation resulted in a significant decrease in the release of dopamine, DOPAC, and HVA. Inflammation also decreased the mRNA levels of the D1R suggesting that inflammation negatively regulates dopamine signaling (Coffeen et al., 2010). In our previous studies of methamphetamine using a focal embolic stroke model we found that low dose methamphetamine exerted a neuroprotective effect that was dependent on a PI3K/AKT pathway. Interestingly, activation of the PI3K/AKT pathway, in addition to suppressing apoptotic factors, is necessary for activation of nuclear factor erythroid 2-related factor 2 antioxidant response element (Nrf2-ARE). Nrf2-ARE is commonly involved in the transcriptional regulation of genes encoding antioxidant proteins such as glutathione (GSH) under stress conditions (Chen et al., 2009; Jin et al., 2008). Thus, increasing the presence and activity of Nrf2-ARE through AKT would likely decrease ROS production after TBI, and there is a growing body of evidence indicating that ROS directly affects the expression of pro-inflammatory genes. Gupta et al. (2012) demonstrated that ROS activate various transcription factors such as nuclear factor kappa-light-chain-enhancer of activated B cells [NF-κB], activator protein-1, hypoxia-inducible factor-1α, and signal transducer and activator of transcription 3, resulting in the expression of proteins that control inflammation, (Gupta et al., 2012). While we do not know if a methamphetamine-dopamine-PI3K/AKT pathway is activating Nrf2-ARE leading to decreased ROS, the potential neuroprotective pathway warrants future investigation. An Nrf2-ARE mediated decrease in ROS may be altering expression of the pro-inflammatory genes we observed in the cortex of methamphetamine treated TBI animals. Testing these hypotheses in future studies will hopefully elucidate the mechanism(s) by which methamphetamine reduces cognitive and neurological impairment after TBI.

We also observed additional changes in the gene expression profile that further suggest methamphetamine treatment impacts multiple pathophysiological pathways. For example, we detected a 2-fold increase in the expression of corticotropin-releasing hormone (CRH) in the brains of methamphetamine treated rats following TBI, compared to a 1.7 fold decrease in saline treated controls. CRH has been shown to protect primary cortical and hippocampal neurons from apoptosis when administered 8 h after an acute neurological insult (Stevens et al., 2003). The in-vivo administration of CRH after global ischemia in Wistar rats increased survival of CA1 neurons by 61% (Charron et al., 2008). Similar to our observations here, Charron et al. (2008) demonstrated that CRH treatment could be delayed until 8 h after ischemic injury and that treatment significantly improved spatial memory and preserved CA1 neuronal survival (Charron et al., 2008). Finally,

Koutmani et al. (2013) recently demonstrated that CRH promotes the proliferation and survival of neuronal progenitor cells (Koutmani et al., 2013). This is noteworthy as we recently demonstrated that methamphetamine treated rats have significantly more doublecortin positive, immature neurons within the subgranular zone of the dentate gyrus after TBI compared to saline treated controls (Administration F.a.D, 2007). This suggests that methamphetamine may promote proliferation and survival of neuronal precursors through the activity of CRH.

Conclusion

The data presented in this study indicate that low dose methamphetamine is neuroprotective when delivered 8–12 h after a severe TBI in a rodent model. In addition to reducing neurological and cognitive impairment, methamphetamine reduced neuronal death in the hippocampus when delivered up to 12 h after TBI. These data, combined with our previously published studies, suggest a novel, neuroprotective use for methamphetamine.

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